

**Molecular Bases of Disease:**  
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# Elongation Factor-1 $\alpha$ Is a Novel Protein Associated with Host Cell Invasion and a Potential Protective Antigen of *Cryptosporidium parvum*\*

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**Background:** Apicomplexan parasites, including *Cryptosporidium*, possess organelles associated with host cell invasion.

**Results:** A chicken-derived monoclonal antibody inhibited host cell invasion by *C. parvum*. The corresponding antigen, which localizes to the apical region of the parasite, was identified as elongation factor-1 $\alpha$  (EF-1 $\alpha$ ).

**Conclusion:** EF-1 $\alpha$  mediates cryptosporidial cytoskeletal complex critical for host cell invasion.

**Significance:** The findings suggest that cryptosporidial EF-1 $\alpha$  could serve as a novel anti-infective target antigen.

The phylum Apicomplexa comprises obligate intracellular parasites that infect vertebrates. All invasive forms of Apicomplexa possess an apical complex, a unique assembly of organelles localized to the anterior end of the cell and involved in host cell invasion. Previously, we generated a chicken monoclonal antibody (mAb), 6D-12-G10, with specificity for an antigen located in the apical cytoskeleton of *Eimeria acervulina* sporozoites. This antigen was highly conserved among Apicomplexan parasites, including other *Eimeria* spp., *Toxoplasma*, *Neospora*, and *Cryptosporidium*. In the present study, we identified the apical cytoskeletal antigen of *Cryptosporidium parvum* (*C. parvum*) and further characterized this antigen in *C. parvum* to assess its potential as a target molecule against cryptosporidiosis. Indirect immunofluorescence demonstrated that the reactivity of 6D-12-G10 with *C. parvum* sporozoites was similar to those of anti- $\beta$ - and anti- $\gamma$ -tubulins antibodies. Immunoelectron microscopy with the 6D-12-G10 mAb detected the antigen both on the sporozoite surface and underneath the inner membrane at the apical region of zoites. The 6D-12-G10 mAb significantly inhibited *in vitro* host cell invasion by *C. parvum*. MALDI-TOF/MS and LC-MS/MS analysis of tryptic peptides revealed that the mAb 6D-12-G10 target antigen was elongation factor-1 $\alpha$  (EF-1 $\alpha$ ). These results indicate that *C. parvum* EF-1 $\alpha$  plays an essential role in mediating host cell entry by the parasite and, as such, could be a candidate vaccine antigen against cryptosporidiosis.

*Cryptosporidium* parasites (Protozoa, Apicomplexa) are recognized as significant pathogens of both immunocompetent and immunocompromised vertebrate hosts worldwide, and are of increasing clinical significance in humans, livestock, birds, and wildlife. Parasites infect epithelial cells lining the luminal surfaces of the digestive or respiratory tracts (1); the resulting clinical signs include diarrhea, leading to wasting and eventual death in immunocompromised patients (2). Despite the magnitude and severity of cryptosporidial infections, there are currently no effective treatments such as approved vaccines, immunotherapies, or parasite-specific pharmaceuticals against cryptosporidiosis (3, 4).

All invasive forms of Apicomplexans (referred as zoites) including *Cryptosporidium* spp., possess a unique complex of organelles located at the anterior end of the organism (the apical complex). The apical complex comprises rhoptries, micronemes, and dense granules and, additionally, an apical assembly of cytoskeleton-associated structures such as the conoid, polar/apical rings, and microtubular protrusions. The apical complex of zoites of *Cryptosporidium* spp. (5–8) and other closely related Apicomplexans (9–13) are involved in parasite attachment, invasion, and intracellular development. Thus, these organelles and their molecular constituents are thought to provide rational targets for immunological therapy or drug treatment to control infections by these parasites.

The chicken antibodies have been demonstrated to be useful for immunochemical research and clinical applications. In contrast to mammals, chicken antibody diversity is mostly generated by somatic mechanisms (14–16). Thus, it may be possible to produce antibodies in chickens that are difficult or impossible to produce in mammals (17). To this end, we have previously developed chicken monoclonal antibodies (mAbs) raised

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against *Eimeria acervulina* (Protozoa, Apicomplexa) and demonstrated that one such mAb (6D-12-G10) recognized the conoid of *E. acervulina* sporozoites and significantly inhibited sporozoite invasions of T lymphocytes *in vitro* (18). Furthermore, mAb 6D-12-G10 showed high cross-reactivity with related parasites, including other *Eimeria* spp., *Neospora caninum*, *Toxoplasma gondii*, *Cryptosporidium parvum*, and *C. muris* (19, 20). Indirect immunofluorescent assay revealed that mAb 6D-12-G10 is reactive only with the apical region of the zoites. These results provide convincing evidence that the antigen recognized by mAb 6D-12-G10 is a highly conserved epitope and thus a novel target vaccine antigen against many infections caused by apicomplexa parasites including *Cryptosporidium*.

In the present study, we show evidence that the epitope recognized in *C. parvum* by mAb 6D-12-G10 was a protein of ~48 kDa related to cytoskeletal function. Immunofluorescence staining and Western blotting patterns of mAb 6D-12-G10 were similar to, yet clearly distinct from, those of known cytoskeletal proteins. Immunoelectron microscopy confirmed the apical cytoskeletal location of the antigen recognized by 6D-12-G10. MALDI-TOF/MS and LC-MS/MS analyses of the target identified the protein as *C. parvum* elongation factor-1 $\alpha$  (EF-1 $\alpha$ ).<sup>2</sup>

## EXPERIMENTAL PROCEDURES

**Parasites**—*C. parvum* oocysts, strain HNJ-1, were originally obtained from the feces of a patient in Japan (21) and passaged in severe combined immunodeficiency mice at Osaka City University, Japan. Oocysts were purified by sugar flotation and stored at 4 °C in phosphate-buffered saline (PBS), pH 7.2, for not more than 1 month before use or before freeze-drying for gel electrophoresis. *C. parvum* sporozoites and merozoites were prepared as previously reported (20).

**Antibodies**—Cell culture supernatant containing mAb 6D-12-G10 was used (18). Rabbit anti-actin polyclonal Ab against a synthetic peptide from the NH<sub>2</sub>-terminal region (A5060, Sigma), mouse anti-myosin mAb against the myosin heavy chain polypeptide of human uterus smooth muscle extract (M7786, Sigma), mouse anti- $\alpha$ -tubulin mAb against the COOH-terminal region of  $\alpha$ -tubulin isoform of sea urchin sperm axonemes (T5168, Sigma), mouse anti- $\beta$ -tubulin mAb against purified rat brain tubulin (T4026, Sigma), and mouse anti- $\gamma$ -tubulin mAb against a synthetic peptide from the NH<sub>2</sub>-terminal region (T6557, Sigma) were used. All Abs were used at 1:100, except for anti- $\alpha$ -tubulin Ab (used at 1:1,000). A rabbit anti-*C. parvum* antiserum was raised against soluble antigens and recognized many life cycle stages of the parasite, including oocyst walls and surface membranes of immature meronts, sporozoites, and merozoites (22). This Ab was used to detect the parasites in cell invasion inhibition assay, as described below.

**Western Blot Analysis**—One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as previously reported (20), except that horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG

(1:10,000, Sigma) or HRP-conjugated goat anti-rabbit IgG (1:5,000, Sigma) were used as secondary Abs. As a control, purified normal rabbit IgG (Sigma) or normal mouse IgG (Sigma) (at a 1:1,000 dilution) was used.

**Pronase Digestion**—A whole sporozoite extract was prepared by sonication of *C. parvum* in PBS (20). Twenty micrograms of the extract was treated with Pronase (Sigma) or vehicle control at a 1:1 (w:w) ratio and digested at 37 °C for 2 h in 100 mM Tris-HCl (Nacalai Tesque) and 10 mM CaCl<sub>2</sub> (Sigma), pH 8.0, as previously described (23). The samples were analyzed by Western blotting using mAb 6D-12-G10 as described above.

To confirm reactivity against the Pronase-digested extract, the Pronase- or mock-digested sporozoite extract (50  $\mu$ l/well at 50  $\mu$ g/ml) was coated onto flat-bottomed 96-well enzyme-linked immunosorbent assay (ELISA) plates (BD Biosciences). Nonspecific binding was blocked by incubating the plates with 1% bovine serum albumin in PBS for 1 h at 37 °C. mAb 6D-12-G10 or control (purified normal chicken IgG (1:1,000, Sigma)) was diluted in Iscove's modified Dulbecco's medium, and distributed at 50  $\mu$ l/well. Following incubation at 37 °C for 1 h, plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST) and then incubated at 37 °C for 1 h with 50  $\mu$ l/well of HRP-labeled rabbit anti-chicken IgG F(ab')<sub>2</sub> fragment (1:1,000, Cortex Biochemistry, CA). Plates were again washed 3 times with PBST and developed using TMB microwell peroxidase substrate (KPL) according to the manufacturer's instructions. The single wavelength absorbance was measured at 450 nm by an ELISA plate reader (ARVO-2, PerkinElmer Life Sciences). The mean optical density value was calculated for reactions performed in triplicate. Statistical analysis was performed using Student's *t* test; *p* values of 0.05 or below were considered significant.

**Indirect Immunofluorescence Assay**—*C. parvum* sporozoites were dried on glass slides as previously reported (20), fixed in absolute methanol, and stored at -80 °C until use. All subsequent steps were performed at room temperature. To extract the detergent-soluble proteins associated with the surface membrane of sporozoites, the cells were permeabilized by incubation for 20 min in PBS containing 0, 0.1, or 1% Triton X-100 (Sigma). After washing 3 times with PBS, the slides were incubated for 40 min with 50  $\mu$ l of mAb 6D-12-G10 or negative control (purified normal chicken IgG (Sigma) diluted in Iscove's modified Dulbecco's medium). The slides were washed 3 times with PBST, and then incubated for 30 min with 50  $\mu$ l of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken IgG (1:1,000, Sigma). After washing with PBST, the slides were mounted with 50% glycerol (Nacalai Tesque) in PBS and examined using a epifluorescence microscope (ECLIPS E600, Nikon).

Indirect immunofluorescence assay of cytoskeletal antigens of *C. parvum* sporozoites was conducted as reported previously (24) with the following modifications. Permeabilization was performed using 0.1% Triton X-100, and reactions were performed using anti-actin, anti-myosin, anti- $\alpha$ -tubulin, anti- $\beta$ -tubulin, and anti- $\gamma$ -tubulin as primary Abs. Controls were performed using purified normal rabbit IgG (Sigma) or normal mouse IgG (Sigma) (1:1,000). Secondary Abs were consisted of

<sup>2</sup> The abbreviations used are: EF-1 $\alpha$ , elongation factor-1 $\alpha$ ; PB, phosphate buffer; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; LC-MS/MS, liquid chromatography/tandem mass spectrometry.



FITC-conjugated goat anti-rabbit IgG (Sigma) or FITC-conjugated goat anti-mouse IgG F(ab')<sub>2</sub> fragment (Sigma) diluted 1:100.

**Two-dimensional Polyacrylamide Gel Electrophoresis**—Two-dimensional PAGE was performed using the Protean IEF Cell (Bio-Rad) according to the manufacturer's protocol. Briefly,  $1.0 \times 10^9$  freeze-dried *C. parvum* oocysts were dissolved in 1.0–2.0 ml of rehydration/sample buffer containing 8 M urea, 2.0% CHAPS, 50 mM dithiothreitol, 0.2% Bio-lyte 3/10, and 0.001% bromophenol blue. The solution was sonicated in an ice bath (Taitec, Saitama) and centrifuged at  $600 \times g$  for 5 min; the supernatant was aliquoted and stored at  $-80^\circ\text{C}$  until use. The protein concentration was determined using the Coomassie Blue Assay Kit (Pierce). For first-dimension electrophoresis, ReadyStrip<sup>TM</sup> pH 3–10 IPG strips (7 cm, Bio-Rad) were rehydrated for 12 h with 100  $\mu\text{l}$  of 200  $\mu\text{g}/\text{ml}$  of oocyst extract. Focusing conditions were 20 min at 250 V, followed by voltage ramping to 4,000 V over a 2-h interval and electrophoresis for 2.5 h at 4,000 V at  $20^\circ\text{C}$ . For second-dimension electrophoresis, proteins were resolved on 4% stacking, 12% gradient SDS-PAGE gels at 200 V. Second-dimension gels were blotted to PVDF membrane (Immobilon Transfer Membranes, Millipore) and analyzed by Western blotting as described above. For protein identification, the gels were stained using the Silver Stain II Kit (Wako).

**Immunoelectron Microscopy**—*C. parvum* sporozoites ( $1.0 \times 10^7$ ) were excysted as previously reported (20) and resuspended in 400  $\mu\text{l}$  of RPMI 1640 medium. The resulting suspension was applied (at 5.0  $\mu\text{l}/\text{grid}$ ) to carbon-stabilized 200-mesh nickel grids covered with thin collodion film (Nissin EM) and allowed to settle for 20 min in a humidity chamber. Excess fluid was removed and the grids were air-dried. Parasites were stained with mAb 6D-12-G10 or normal chicken IgG (Sigma), washed with PBS, incubated with 10-nm colloidal gold-labeled rabbit anti-chicken IgG (EY Laboratories), washed 3 times with PBS and water, stained for 30 s with 2.0% phosphotungstic acid, pH 7.5 (TAAB Laboratories), and examined using a Hitachi H-7500 electron microscope (Hitachi Science Systems). In some experiments, the sporozoites were treated with 0.1% Triton X-100 in RPMI 1640 medium for 5–10 min at  $4^\circ\text{C}$ , fixed in 0.1% glutaraldehyde (TAAB Laboratories) and 2.0% paraformaldehyde (Merck) in 0.1 M phosphate buffer (PB) at  $4^\circ\text{C}$  for 4–6 days, and examined by immunoelectron microscopy via the above procedure.

For immunolocalization of the antigen recognized by mAb 6D-12-G10, excysted *C. parvum* sporozoites and merozoites cultured in human colonic tumor cells (HCT-8, Dainippon Pharmaceutical) (20) were fixed with 0.05% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M PB at  $4^\circ\text{C}$  for 30 min, washed in ice-cold PB, dehydrated in a graded series of cold ethanol solutions, and embedded in LR White resin (London Resin). Thin sections were cut and mounted on nickel grids (Nissin EM); the grids then were blocked with 0.1 M glycine (Nacalai Tesque) in PBS followed by 1.0% nonfat dry milk in PBS. Following staining (overnight at  $4^\circ\text{C}$ ) with mAb 6D-12-G10 or normal chicken IgG (Sigma) and colloidal gold-labeled secondary Ab as above, grids were incubated for 20 min in 1.0% uranyl acetate (TAAB Laboratories) and examined as described above.

**Invasion Inhibition Assay**—*C. parvum* oocysts were treated with 10% sodium hypochlorite (Wako) in PBS at  $4^\circ\text{C}$  for 15 min

to excyst sporozoites and washed 2 times with cold PBS. Treated oocysts ( $1.0 \times 10^5$ ) then were mixed with 200  $\mu\text{l}$  of one of the following: mAb 6D-12-G10, an irrelevant mAb (8D-2) raised against *E. acervulina* and known not to react with *Cryptosporidium* parasites (20); or normal chicken IgG (Sigma) and only culture medium. mAbs 6D-12-G10 and 8D-2 were present at approximately equal IgG concentrations in the hybridoma supernatants. Parasite/mAb mixtures were incubated with confluent monolayers of HCT-8 cells in perfusion chambers (CoverWell<sup>TM</sup>, Grace Bio-Labs) at  $37^\circ\text{C}$  for 2 h in 5% CO<sub>2</sub>. Following incubation, the cells were washed 3 times with RPMI 1640 containing 10% FBS (Invitrogen) as culture medium, incubated for an additional 22 h, and fixed with acetone for 10 min. The slides were blocked with 10% normal goat serum (Sigma) and stained with rabbit anti-*C. parvum* antiserum (1:1,000) plus Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (1:1,000, Molecular Probes) as a secondary antibody. The number of intracellular zoites was counted in 6.6-mm<sup>2</sup> fields per chamber at  $\times 400$  magnification using a fluorescent microscope (Nikon). The percent inhibition of infection was calculated by the following formula:  $(1 - [\text{No. zoites after 6D-12-G10, 8D-2 mAb, or normal chicken IgG treatment} / \text{No. zoites after culture medium}]) \times 100\%$ . The mean percent inhibition was calculated based on four replicates each, and values from each treatment were compared using Student's *t* test (as above).

**Protein Identification**—The protein antigen recognized by mAb 6D-12-G10 was identified by NH<sub>2</sub>-terminal amino acid sequencing in combination with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) and liquid chromatography/tandem mass spectrometry (LC-MS/MS). Briefly, for NH<sub>2</sub>-terminal sequencing, proteins in a *C. parvum* oocyst extract were separated by two-dimensional PAGE, blotted to PVDF, and stained with Coomassie Blue (Bio-Rad). The visible spot in the 48-kDa region was excised from the membrane, and the resulting protein was subjected to automated Edman degradation using the Procise 494 HT (Applied Biosystems). For MALDI-TOF/MS and LC-MS/MS, the 48-kDa spot was separated by two-dimensional PAGE as described above, excised from the gel, and stored at  $-20^\circ\text{C}$  until use. Trypsin (Promega) digestion was carried out as described by Shevchenko *et al.* (25). The tryptic digest was adsorbed to a C-18 reverse phase matrix and eluted with 60% acetonitrile containing 0.2% TFA. MALDI-TOF/MS was performed using a Voyager-DE STR instrument (Applied Biosystems); the observed *m/z* values were analyzed using ProFound software (Proteomic Solutions) and peptide mass fingerprints were queried in the NCBI database. For LC-MS/MS, the tryptic digest was resolved by high-performance liquid chromatography on a C-18 column (0.1  $\times$  50 mm; Michrom BioResources) coupled to a Q-TOF2 mass spectrometer equipped with a nanoelectrospray ionization source (Waters Micromass). Positive ion tandem mass spectra were measured and the data were used to search the non-redundant NCBI protein database using the MASCOT software (Matrix Science).

**Cloning and Sequencing of *C. parvum* EF-1 $\alpha$ -encoding Gene**—Total RNA for *C. parvum* oocysts was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. To

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permit cloning of the full-length transcript encoding *C. parvum* EF-1 $\alpha$ , 5' and 3' rapid amplification of cDNA ends was performed using the SMARTer RACE cDNA Amplification Kit (Takara) using primers CpEF1aRA-R1 (5'-CTG GGG CAT CAA TGA CAG TGT AGT GGT A-3') for the 5' end and CpEF1aRA-F1 (5'-TGT TAC CTT CGC TCC AGC TGG TGT TAC C-3') for the 3' end. The putative signal sequence and membrane topology of the predicted *C. parvum* EF-1 $\alpha$  protein were analyzed using the prediction server SignalP version 4.1 (26) and the TMHMM Server version 2.0, respectively. Sequence analysis of the rapid amplification of cDNA ends products permitted identification and cloning of the complete coding region of the *C. parvum* EF-1 $\alpha$ -encoding transcript.

**Expression of *C. parvum* EF-1 $\alpha$** —Single-stranded cDNA for *C. parvum* mRNA was prepared using the Takara RNA PCR Kit (AMV) version 3.0 (Takara) following the manufacturer's instructions. The recombinant *C. parvum* EF-1 $\alpha$  protein was produced by Bex Co. Ltd. Briefly, the open reading frame (ORF) encoding *C. parvum* EF-1 $\alpha$  was amplified from cDNA from *C. parvum* oocysts by PCR with a pair of primers (forward, EF1 $\alpha$ -BamHI-F (5'-ATT ATT GGA TCC GAT GGG TAA GGA AAA GAC TCA-3'), reverse, EF1 $\alpha$ -XhoI-R (5'-ATT ATT CTC GAG TTA CTT CTT CTT GGA AGT GG-3')) that provided flanking BamHI and XhoI restriction sites for cloning (using a DNA ligation kit) into the pET-47b(+) vector (Novagen). The sequence of the *C. parvum* EF-1 $\alpha$ -encoding construct was confirmed using internal primers. The resulting plasmid was transformed into competent cells of *Escherichia coli* strain BL21 (Takara) following standard methods. The *C. parvum* EF-1 $\alpha$  was successfully expressed with a polyhistidine tag in *E. coli* and purified as soluble protein from bacterial cultures using ProBond<sup>TM</sup> resin (Invitrogen). Purified recombinant protein was electrophoresed on SDS-PAGE gel and detected as an ~50 kDa fusion protein. Reactivity with mAb 6D-12-G10 was assessed by Western blotting.

**Synthesis of *C. parvum* EF-1 $\alpha$  Peptides and Anti-peptide Ab Preparation**—Potential antigenic sites of *C. parvum* EF-1 $\alpha$  were identified using Lasergene DNA program version 6.1 (DNA Star). Six 14-residue peptides (<sup>39</sup>IEKFEKESSEMGGK<sup>52</sup>, <sup>152</sup>KMDTCEYKQSRFDE<sup>165</sup>, <sup>196</sup>NMVERSDKMPWYKG<sup>209</sup>, <sup>217</sup>DTMEPPKRPTDKPL<sup>230</sup>, <sup>312</sup>DAKNDDPAKGCEFT<sup>325</sup>, and <sup>363</sup>MDKRSQGVLEENPK<sup>376</sup>) suggested based on predicted antigenicity were synthesized on a ABI433A peptide synthesizer (Applied Biosystems) by Scrum Inc. Peptides were deprotected and lyophilized, and their purity was assessed using an Alliance HPLC System (Waters Corporation) and MALDI-TOF/MS. The reactivity of mAb 6D-12-G10 with these peptides was assessed by ELISA as described above.

A polyclonal antibody against *C. parvum* EF-1 $\alpha$  was generated by Scrum Inc. using the synthetic peptide, <sup>217</sup>DTMEPPKRPTDKPL<sup>230</sup>, with the highest antigenicity as predicted by the Lasergene DNA program. Briefly, this peptide was coupled to keyhole limpet hemocyanin and administered in rabbit by serial immunization at 0.25 mg/dose (initial intradermal injection in complete Freund's adjuvant, followed by four subcutaneous booster injections in incomplete Freund's adjuvant at intervals of 2 weeks). Immune sera were collected on day

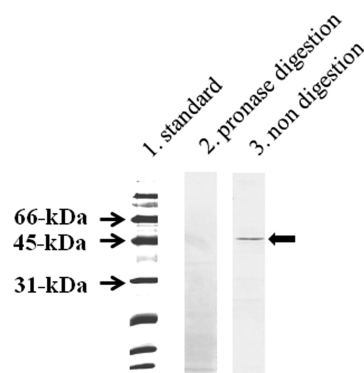


FIGURE 1. Western blot analysis (using mAb 6D-12-G10) of *C. parvum*-soluble antigens treated with Pronase (lane 2) or vehicle control (mock digest) (lane 3). The arrow indicates the 48-kDa band recognized by mAb 6D-12-G10. Lane 1, molecular mass standards.

63, i.e. 1 week after the final booster shot. Serum reactivity against *C. parvum* was determined by Western blotting.

## RESULTS

**Pronase Sensitivity of the Epitope Recognized by mAb 6D-12-G10**—In our initial attempt to characterize the antigen recognized by mAb 6D-12-G10, soluble sporozoites were disrupted by sonication; the soluble protein fraction was treated with Pronase or vehicle control and analyzed by Western blotting. As shown in Fig. 1, a 48-kDa band was detected in the mock-digested sample, but was not observed after Pronase treatment. Furthermore, similar Pronase sensitivity was observed via ELISA: the optical density value of antigens treated with Pronase ( $0.033 \pm 0.027$ ) was significantly decreased compared with those of mock-digested ( $1.201 \pm 0.213$ ) and untreated ( $1.309 \pm 0.228$ ) *C. parvum* antigens.

**Reactivity of mAb 6D-12-G10 with Triton X-100-treated Sporozoites**—Fig. 2A shows immunofluorescence staining of *C. parvum* sporozoites treated with 0.1% Triton X-100. Notably, 20 min pre-treatment of whole cells with 0, 0.1, or 1% Triton X-100 did not alter the reactivity with mAb 6D-12-G10, and positive reactions were observed only at the apical region of the sporozoites.

**Comparison of mAb 6D-12-G10 with Other Cytoskeleton-specific Abs**—Our previous results demonstrated that the antigen recognized by mAb 6D-12-G10 was located in the apical cytoskeleton of *E. acervulina* sporozoites (18). Therefore, we examined the location of the cross-reacting antigen of *C. parvum* in relationship to that of other cytoskeletal proteins by indirect immunofluorescence and Western blot analysis. Abs against actin, myosin,  $\beta$ -tubulin, and  $\gamma$ -tubulin stained only the apical region of the sporozoites (Fig. 2, C, E, I, and K). The antigenic structures recognized by the individual Abs were localized as follows: anti-actin Ab, within the internal portion of the apical region; anti-myosin Ab, at the tip of the apical region; anti- $\beta$ - and  $\gamma$ -tubulin Abs, at the surface of the apical region; and anti- $\alpha$ -tubulin Ab, at the apical region and along the central microtubules (Fig. 2, C, E, I, K, and G, respectively). Labeling by the mAb 6D-12-G10 was similar with that seen with the anti- $\beta$ -tubulin and anti- $\gamma$ -tubulin Abs. In control assays, normal rabbit and mouse IgG did not provide labeling (data not shown).

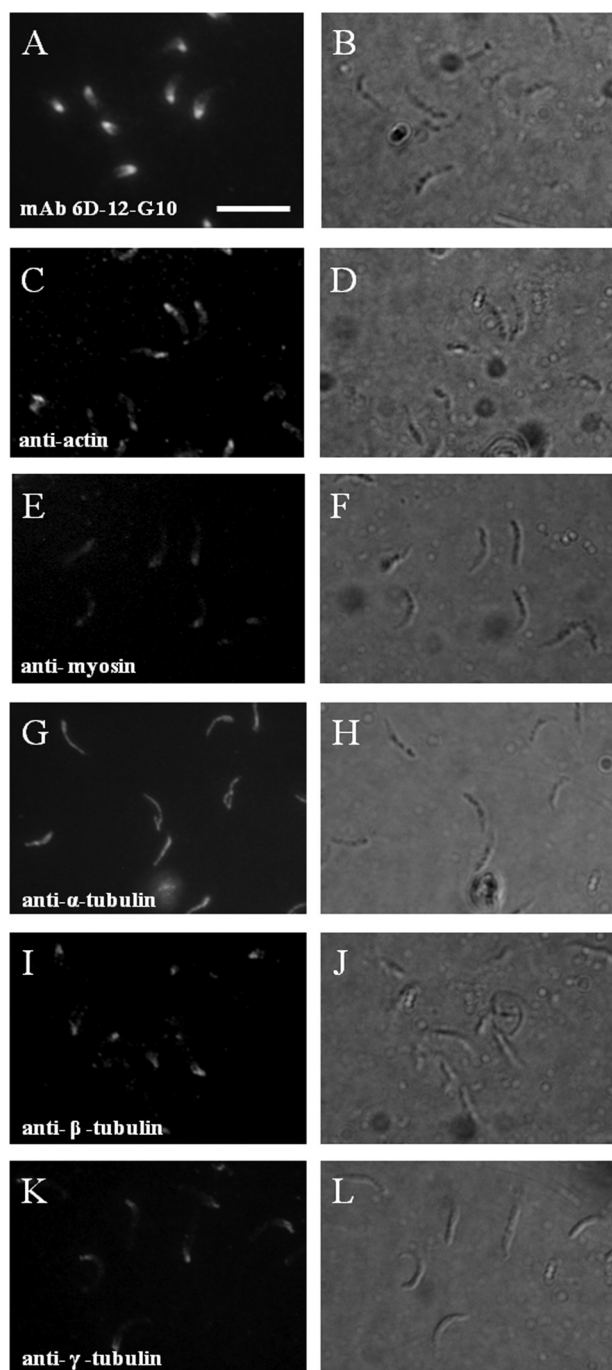


FIGURE 2. Photomicrographs of *C. parvum* sporozoites of immunofluorescence staining (left panels) and bright field by interference contrast microscopy (right panels). A and B, mAb 6D-12-G10; C and D, anti-actin Ab; E and F, anti-myosin Ab; G and H, anti- $\alpha$ -tubulin Ab; I and J, anti- $\beta$ -tubulin Ab; K and L, anti- $\gamma$ -tubulin Ab. Bar = 10  $\mu$ m.

In Western blot analyses, mAb 6D-12-G10 and Abs against actin and  $\alpha$ -tubulin reacted with protein bands of ~48, 42, and 50 kDa, respectively (Fig. 3). None of the other cytoskeleton-specific Abs or controls detected individual bands in Western blots. Immunoblot analysis following two-dimensional PAGE resolution of *C. parvum* proteins revealed that the 48-kDa band recognized by mAb 6D-12-G10 was focused at an isoelectric point (pI) of 9.74 (Fig. 4A). The actin and  $\alpha$ -tubulin of *C. parvum* were detected at pI values of 5.93 and 6.24, respectively (Fig. 4, B and C).

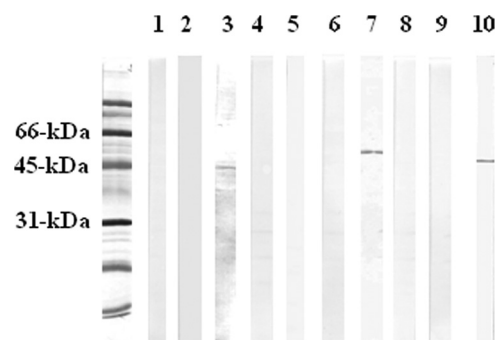


FIGURE 3. One-dimensional Western blot analysis of *C. parvum* antigens. Individual strips from the same gel transfer were subjected to immunodetection using the following antibody or control reagent: lane 1, 1% BSA in PBS (control for HRP-conjugated anti-mouse IgG); lane 2, normal rabbit IgG; lane 3, anti-actin Ab; lane 4, 1% BSA in PBS (control for HRP-conjugated anti-rabbit IgG); lane 5, normal mouse IgG; lane 6, anti-myosin Ab; lane 7, anti- $\alpha$ -tubulin Ab; lane 8, anti- $\beta$ -tubulin Ab; lane 9, anti- $\gamma$ -tubulin Ab; lane 10, mAb 6D-12-G10. The left-most column provides the molecular mass standards.

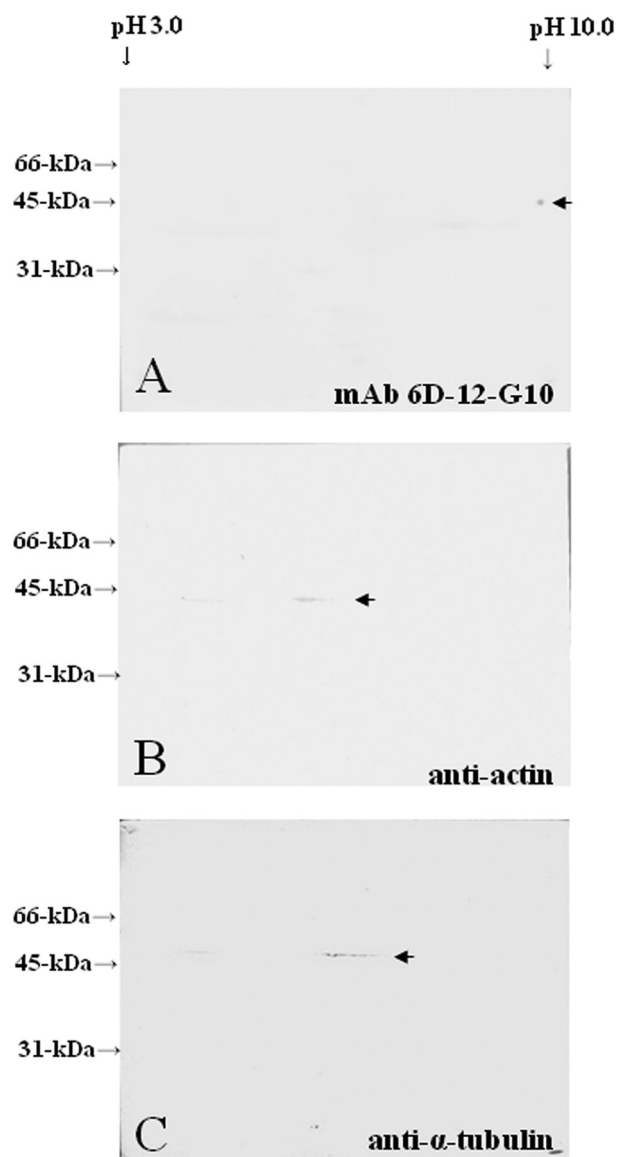


FIGURE 4. Two-dimensional Western blot analysis of *C. parvum* antigens stained with (A) mAb 6D-12-G10, (B) anti-actin Ab, or (C) anti- $\alpha$ -tubulin Ab. The positions of molecular mass standards are indicated on the left. pH values are indicated at the top.



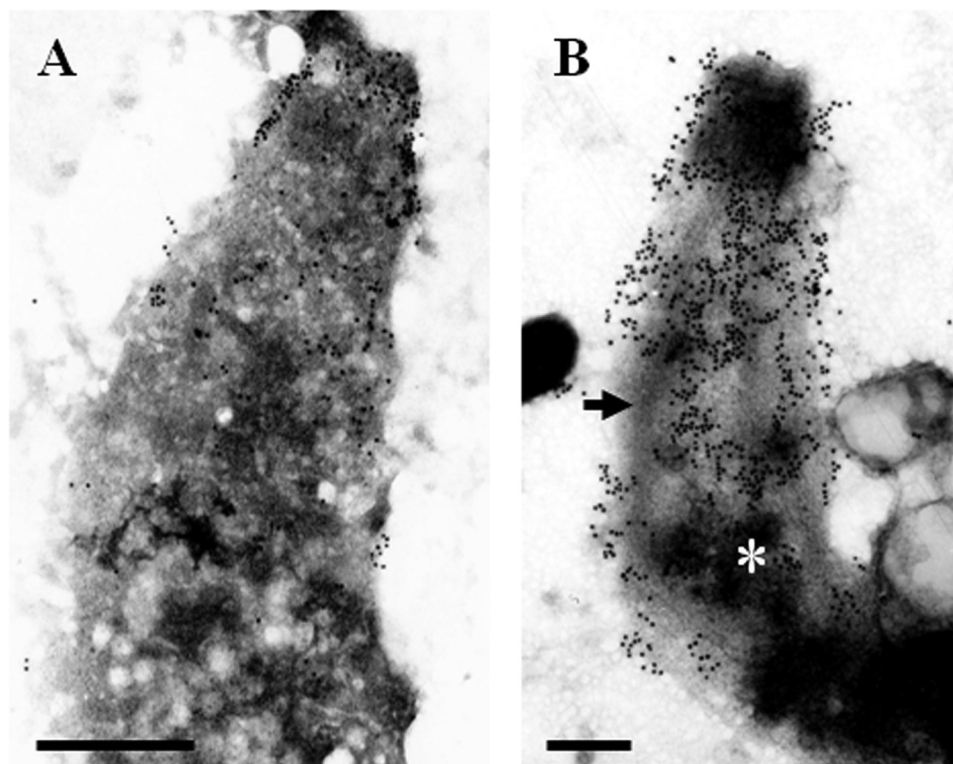


FIGURE 5. Immunogold labeling of *C. parvum* sporozoites with mAb 6D-12-G10. A, untreated sporozoites. B, sporozoites treated with 0.1% Triton X-100. Arrow and asterisk indicate central microtubules and micronemes, respectively. Bar = 0.5  $\mu$ m.

**Immunoelectron Microscopy of the 6D-12-G10 mAb-reactive Antigen at the Apical Region of Zoites**—Immunoelectron microscopy using the 6D-12-G10 mAb with *C. parvum* sporozoites revealed localization of gold particles at the apical region of the parasite surface (Fig. 5A). After treatment of the sporozoites with 0.1% Triton X-100, increased numbers of gold particles were observed across a larger area of the apical region (Fig. 5B). However, the internal organelles (central microtubules and micronemes) were not labeled by mAb 6D-12-G10. Furthermore, mAb 6D-12-G10-targeted gold particles were localized below the inner membrane of the apical region as well as on the surface membrane. In a control assay, normal chicken IgG did not react with the untreated or Triton X-100-treated sporozoites (data not shown).

Immunoelectron microscopy of longitudinal sections of *C. parvum* sporozoites and merozoites using mAb 6D-12-G10 revealed localization of gold particles close to the surface membrane at the apical region; labeling was not observed within the internal organelles (rhoptries and micronemes) (Fig. 6, A and B). On sporozoites, gold particles were observed between the surface membranes and beneath the inner membrane (Fig. 6C). Gold particles were not present in the immature trophozoites (Fig. 6D), but were observed at the apical region as well as the surface regions and beneath the inner membrane of mature merozoites (Fig. 6, E and F). In control assays, normal chicken IgG did not react with sporozoites, trophozoites, or merozoites.

**mAb 6D-12-G10 Blocking of Host Cell Invasion by *C. parvum***—The 6D-12-G10 mAb was originally identified by its ability to block *E. acervulina* sporozoite invasion of chicken CD8<sup>+</sup> T lymphocytes (18). Therefore, we next assessed the ability of this

mAb to inhibit host cell invasion by *C. parvum*. As shown in Fig. 7, mAb 6D-12-G10 blocked *C. parvum* infection of HCT-8 cells by 80.9% compared with culture medium. In a control assay, normal chicken IgG, and mAb 8D-2 (a chicken mAb that reacts with *E. acervulina* but not *C. parvum*) inhibited invasion by 7.9 and 51.2%, respectively. In this assay, mAb 8D-2 serves as an appropriate control because both 8D-2 and 6D-12-G10 are derived as cell culture supernatants from confluent chicken hybridomas. Although the culture supernatant compared with fresh culture medium (only culture medium or normal chicken IgG) could reduce the viability itself of HCT-8 host cells resulting in a decrease of the infection (data not shown), the greater inhibition by mAb 6D-12-G10 compared with 8D-2 was significant ( $p < 0.01$ ).

**Identification of EF-1 $\alpha$  as the Protein Recognized by mAb 6D-12-G10**—Silver staining of the two-dimensional PAGE-derived immunoblot confirmed that the 48-kDa protein recognized by mAb 6D-12-G10 ran as a single spot at basic pH (Fig. 8A). NH<sub>2</sub>-terminal amino acid sequence analysis of the spot recovered from the two-dimensional PAGE-blotted membrane of the *C. parvum* extract was unsuccessful, suggesting that the NH<sub>2</sub> terminus was blocked (data not shown). Tryptic peptides derived from the 6D-12-G10 mAb-reactive protein were analyzed by MALDI-TOF/MS and LC-MS/MS. The results of database searching of the tandem mass spectra with the Mascot Search Program are shown in Tables 1 and 2. MALDI-TOF/MS and LC-MS/MS demonstrated that the tryptic peptides provided, respectively, 40–49 and 55% sequence coverage of the predicted *C. parvum* EF-1 $\alpha$  protein. The extent of the coverage by LC-MS/MS is indicated in Fig. 8B. Based on these sequence

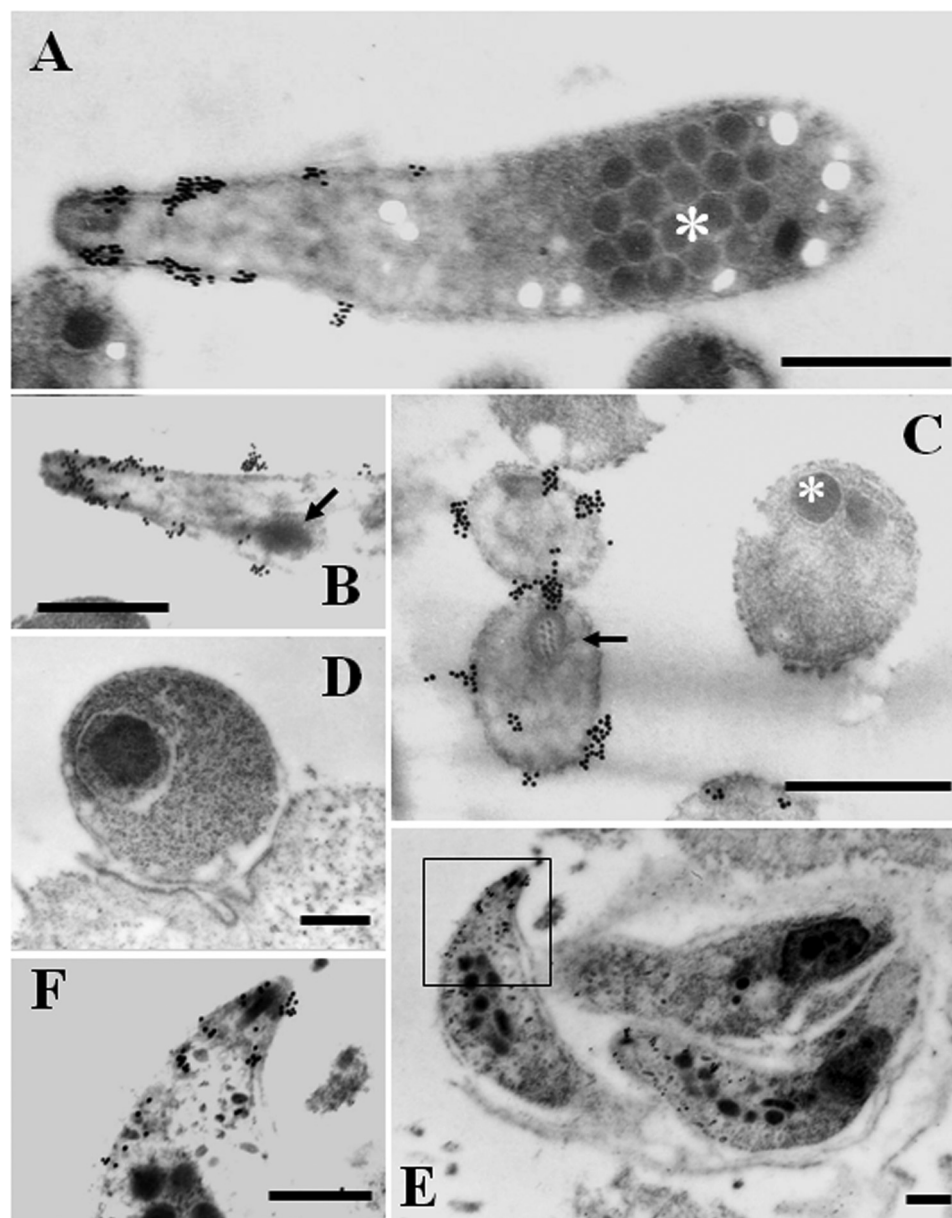


FIGURE 6. Immunogold labeling of thin sections of *C. parvum* with mAb 6D-12-G10. *A*, longitudinal section of sporozoite. *B*, enlarged area of the apical region of a sporozoite. *C*, traverse section of a sporozoite. *D*, trophozoite within HCT-8 cells. *E*, merozoite within mature meront. *F*, enlarged magnification of the apical region of merozoite in the boxed regions in *E*. Arrows and asterisks show rhoptries and micronemes, respectively. Bar = 0.5  $\mu$ m.

alignments, the probability-based Mowse score was 794 for EF-1 $\alpha$  and 135 for the *C. parvum* extracellular protein with a signal peptide (CpCCP2), the second most likely identification (Table 2). However, the theoretical molecular mass (184.41-kDa) and pI (6.36) of CpCCP2 were inconsistent with the observed values, therefore suggesting that EF-1 $\alpha$  is the protein recognized by mAb 6D-12-G10.

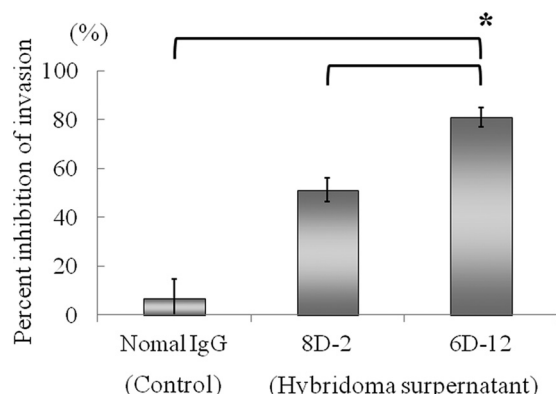
**Cloning of *C. parvum* EF-1 $\alpha$ -encoding Gene and Validation of Reactivity of the Recombinant Protein with mAb 6D-12-G10**—Sequence analysis of the rapid amplification of cDNA ends products permitted cloning of a 1,308-bp *C. parvum* transcript encoding EF-1 $\alpha$ . All sequences were identical with those previously reported in GenBank<sup>TM</sup> (accession numbers CAD98440 and AAC02806), and included a 435-codon ORF flanked by  $\sim$ 100 and 300 bp of non-coding 5' and 3' sequences, respec-

tively. The encoded protein has a predicted molecular weight of  $48.16 \times 10^3$  and a pI of 8.95, and but is not expected to harbor signal peptide or transmembrane domains. However, as assayed by Western blotting, mAb 6D-12-G10 showed no reactivity with the recombinant *C. parvum* EF-1 $\alpha$  expressed in *E. coli* (data not shown).

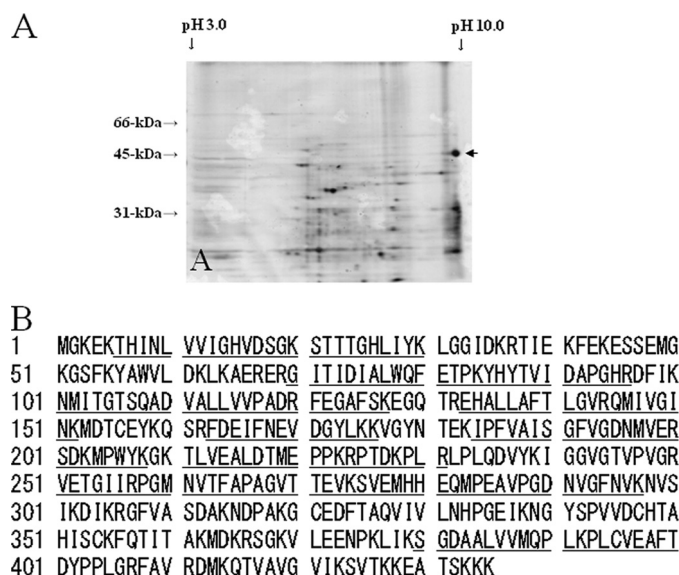
**mAb 6D-12-G10 Detection of Synthetic Peptides of *C. parvum* EF-1 $\alpha$** —By ELISA, mAb 6D-12-G10 did not show specificity for any of 6 synthetic peptides generated on the basis of the EF-1 $\alpha$  protein sequence (data not shown). Thus, the epitope recognized by this mAb was not provided by these amino acid sequences. A polyclonal anti-peptide Ab raised against the DTMEPPKRPTDKPL peptide detected a protein in *C. parvum* extracts that was indistinguishable (in molecular weight and pI) from that recognized by mAb 6D-12-G10 (Fig. 9). Unfortu-



# Elongation Factor-1 $\alpha$ Is Associated with Host Cell Invasion of *C. parvum*



**FIGURE 7. *In vitro* inhibition of *C. parvum* sporozoite invasion of HCT-8 cells by mAb 6D-12-G10.** Invasion inhibition by mAb 6D-12-G10 was compared with those of normal chicken IgG and the irrelevant mAb 8D-2. Each bar represents the mean  $\pm$  S.D. of 4 experiments. The asterisk indicates significantly increased inhibition of invasion by mAb 6D-12-G10 compared with normal chicken IgG and mAb 8D-2 ( $p < 0.01$ ).



**FIGURE 8. Identification of the mAb 6D-12-G10-reactive antigen.** A, silver-stained two-dimensional gel electrophoresis product. Arrow indicates the position of the antigen spot recognized by mAb 6D-12-G10. The positions of molecular mass standards are indicated on the left. pH values are indicated at the top. B, amino acid sequence of *C. parvum* EF-1 $\alpha$  (NCBI database accession no. CAD98440). Tryptic peptides identified by LC-MS/MS analysis are underlined.

**TABLE 1**

Summary of MALDI-TOF MS analysis of *C. parvum* antigen spot recognized by mAb 6D-12-G10

Protein (organism)	Hits	Database accession No.	% Sequence coverage
Elongation factor 1 $\alpha$ ( <i>C. parvum</i> )	45.01/8.3	AAC02806	49
Elongation factor 1 $\alpha$ ( <i>C. parvum</i> )	48.43/9.1	CAD98440	46
Elongation factor 1 $\alpha$ ( <i>C. parvum</i> )	48.04/9.1	EAL36164	40

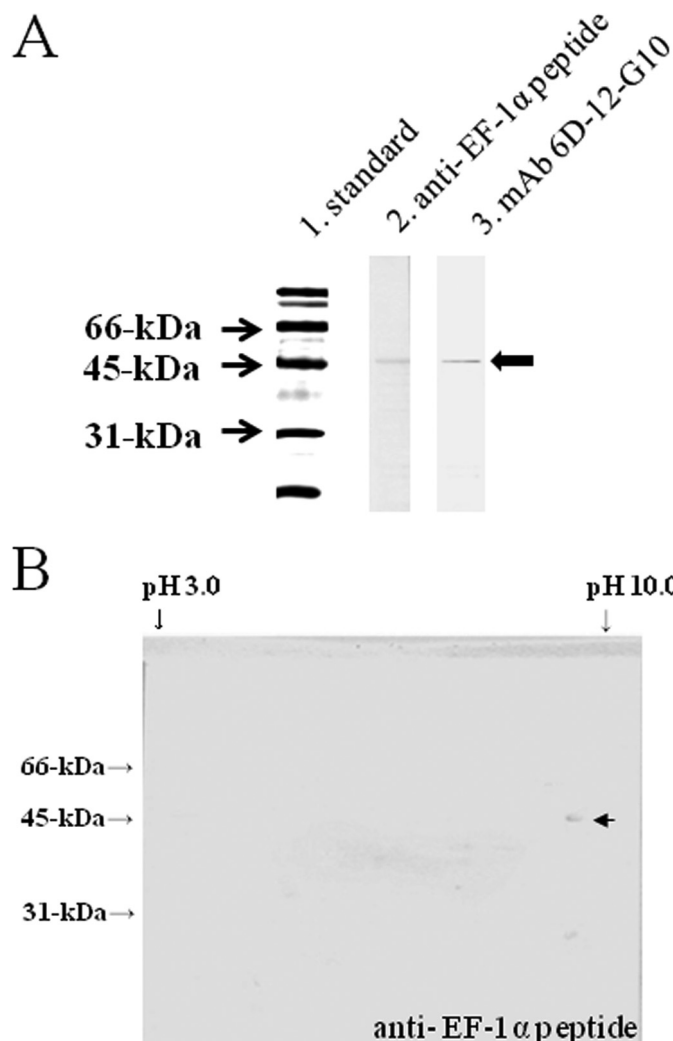
nately, this anti-peptide Ab did not show any specific reactivity with *C. parvum* sporozoites by immunofluorescence staining (data not shown). Probably, the peptide as a epitope might be masked by carbohydrate chain or protein folding. These results indicate that the *C. parvum* antigen recognized by mAb 6D-12-

**TABLE 2**

Summary of LC-MS/MS analysis of *C. parvum* antigen spot recognized by mAb 6D-12-G10

Protein (organism)	Hits	Database accession No.	Probability-based Mowse score <sup>a</sup>	% Sequence coverage
Elongation factor 1 $\alpha$ ( <i>C. parvum</i> )	48.13/8.95	CAD98440	794	55
Extracellular protein with a signal peptide (CpCCP2) ( <i>C. parvum</i> )	184.41/6.36	EAK90522	135	2

<sup>a</sup> The Mowse score is calculated as  $-\log 52(P)$ , where  $P$  is the probability that the observed match is a random event.



**FIGURE 9. One- and two-dimensional Western blot analyses of *C. parvum* antigens using antibody against *C. parvum* EF-1 $\alpha$  peptide (DTMEPPKRPTDKPL) (A and B, respectively).** Lane 1 in A, molecular mass standards. Arrows indicate band (lane 2 in A) or spot detected by the antibody (Fig. B). Lane 3 in A, mAb 6D-12-G10.

G10 is EF-1 $\alpha$ , and the post-translational modification at the step in protein biosynthesis specific for the protozoan *C. parvum*, but not *E. coli*, could be required for generation of the epitope recognized by mAb 6D-12-G10.

## DISCUSSION

As demonstrated here, the previously described mAb 6D-12-G10, which detects an apical complex antigen of *E. acervulina*,

identified a 48-kDa apical membrane protein of *C. parvum*. Furthermore, mAb 6D-12-G10 reactivity remained in the extract of the Triton X-100-treated *C. parvum* sporozoites, suggesting that the antigen recognized by mAb 6D-12-G10 may be associated with the cytoskeleton protein of *C. parvum* sporozoites but not surface membrane.

The ultrastructure of *Cryptosporidium* zoites, including the architecture and components of the cytoskeleton, are not fully understood. It was reported that *C. parvum* sporozoites lacked structures resembling conoids in zoites (27) and that *C. parvum* sporozoites differed from other apicomplexan parasites in their cytoskeletal structure (24). Therefore, to accurately discern the localization of the mAb 6D-12-G10-reactive antigen in *C. parvum* sporozoites, we compared the mAb reactivities by immunofluorescence and Western blot analysis with those of commercial Abs raised against known cytoskeleton components. All of the cytoskeletal antigens were found to be present in *C. parvum* sporozoites, with actin, myosin, and  $\beta$ - and  $\gamma$ -tubulins localized only to the anterior portion of the parasites. However, only the anti-actin and anti- $\beta$ -tubulin Abs were reactive by Western blotting, suggesting that the conformation of the epitopes recognized by the anti-myosin, anti- $\alpha$ -tubulin, and anti- $\gamma$ -tubulin Abs may be altered by the solubilization conditions used for gel electrophoresis. In our hands, the reactivity of mAb 6D-12-G10 against *C. parvum* was similar to that of anti- $\beta$ -tubulin and anti- $\gamma$ -tubulin Abs as assayed by immunofluorescence, but the antigen recognized by the mAb was distinct from actin and  $\alpha$ -tubulin by Western analysis.

Previously, the mAb 6D-12-G10 was shown to recognize the conoid of *E. acervulina* sporozoites (18). However, in the present study, the mAb 6D-12-G10-reactive antigen was ultrastructurally localized both to the surface and underneath the membrane of the parasite in the apical region; the surface-localized antigen was not extracted by Triton X-100 treatment. Furthermore, this antigen was expressed in a stage-specific manner, being present in sporozoites excysed from oocysts and merozoites within mature meronts, but not in immature trophozoites. Thus, these findings suggest that the antigen recognized by mAb 6D-12-G10 is a protein associated (directly or indirectly) with the cytoskeleton of the apical complex on *C. parvum* invasive zoites, and with the conoid of *E. acervulina*. These structures are known to be related to host cell invasion, a function not ascribed to the cytoskeletons itself.

Protein structural analyses identified the 48-kDa antigen as *C. parvum* EF-1 $\alpha$ . To date, only one study has described the cloning and sequencing of a cryptosporidial EF-1 $\alpha$ -encoding gene, although the details of the function or ultrastructural location of the encoded protein were not reported (28). In general, EF-1 $\alpha$  is highly conserved and ubiquitously expressed in all eukaryotic cells (29–32). Functionally, EF-1 $\alpha$  transfers aminoacylated tRNAs to the ribosome A site in a GTP-dependent reaction (33). In addition, EF-1 $\alpha$  appears to have a number of other functions associated with cell growth, motility, protein turnover, and signal transduction (34, 35), more recently DNA replication/repair protein networks (36) and apoptosis (37). Some among these previous studies have demonstrated that EF-1 $\alpha$  can be bound by actin filaments or microtubules as one function (38–44). In particular, EF-1 $\alpha$  was reported to be a

cytoskeleton-binding protein and to play a role in regulating the assembly and cross-linking of actin filaments and microtubules. The results of the present study, combined with those of our previous publication (18), suggest that EF-1 $\alpha$  associates with the cytoskeleton at the apical region, forming an essential component of the invasion apparatus of the parasite. This hypothesis is consistent with the demonstration by other laboratories that host cell invasion by Apicomplexan parasites is an active process mediated by the cytoskeleton of the parasite, and that the host cell need not play an active role in parasite uptake (45, 46). The distinct localization of EF-1 $\alpha$  between *C. parvum* and *E. acervulina* zoites might be related to differences in ultrastructure or mechanism of invasion, given that *C. parvum* sporozoites infect only epithelial cells, whereas *Eimeria* sporozoites invade both T lymphocytes and intestinal epithelial cells.

Further analysis is needed to clarify the molecular role of EF-1 $\alpha$  during parasite attachment and invasion of host cells. However, considering that the mAb 6D-12-G10 reactive antigen is highly conserved among Apicomplexan genera, EF-1 $\alpha$  warrants investigation as a candidate anti-infective vaccine antigen.

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